UNIVERSITY OF PENNSYLVANIA

CARBON DIOXIDE AS A NARCOTIC AGENT

CHARLOTTE HAYWOOD

A THESIS

IN PHYSIOLOGY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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CARBON DIOXIDE AS A NARCOTIC AGENT.

I. The Effect of Carbon Dioxide upon the Fertilized Egg of *Arbacia*.

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The narcosis which may be produced by carbon dioxide seems to have long been known. Pliny the Elder, in his Natural History, remarked that the marble from Memphis, when ground up and used as a liniment with vinegar, had the virtue of rendering insensible parts of the body to be cut or cauterized. In modern times the value of carbon dioxide as an analgesic was recognized soon after Black's discovery of "fixed air," and in 1788 we find Percival recommending "fixed air" for the relief of painful ulcers. Later it was employed as a local anesthetic by Ingenhousz,[1] Beddoes, Simpson, Follin, Brown-Séquard,[2] Gellé,[2] and others. In 1828, eighteen years before Morton's demonstration of ether anesthesia, Hickman[3] appears to have suggested that general anesthesia be induced by inhalations of carbon dioxide, and it was thus used later in the century by Ozanam and by Gréhant, although never extensively. Ozanam seems to have been impressed by the promptness of recovery from the anesthesia produced in this way. At one time it was thought by some that natural sleep and hibernation were the effects of the accumulation of carbon dioxide. This view has been abandoned, but Kidd (1914) has recently with more plausibility suggested a similar explanation for the dormancy of seeds. The work of Cohn (1918) also indicates the possibility that carbon dioxide may play an important part in keeping the spermatozoa of *Arbacia* inactive until their discharge into sea water.

[1] Cited by Herpin (1864).
[2] Cited by Dastre (1890).
[3] Cited by Simpson (1856).

Inasmuch as carbon dioxide is a normal product of metabolism, an understanding of its characteristic effects on living cells and the mechanism by which they are produced is of importance. Unfortunately, most of the studies previously made on its action have been concerned with entire organisms where numerous complicating factors prevent the separation of its more characteristic effects from others of a less fundamental nature. It is the object of the present paper to study in a quantitative way certain aspects of the narcotic effects of carbon dioxide on single cells—the developing egg cells of *Arbacia*—where complicating factors are reduced to a minimum. In particular, an attempt has been made to determine the extent to which such effects are reversible. A second paper [1] deals similarly with a simple tissue—the striated muscle of the frog—and studies upon ciliated epithelium are to be reported elsewhere.

For quantitative work, the developing egg of *Arbacia* proves very satisfactory. Since favorable material shows about 100 per cent. division under normal conditions, any delay in the occurrence of the first cleavage may be measured and used as a suitable quantitative criterion for narcotic or toxic effects upon the activity of the cell. Smith and Clowes (1924) have studied from a somewhat different point of view from that of the present paper the effects of carbon dioxide upon the cleavage of echinoderm eggs and have found that development is inhibited by CO_2 at pH values which in its absence are without effect upon cleavage. The present experiments differ in at least two respects from those of Smith and Clowes. In the first place, they have to do with exposures of varying lengths *followed by a return to normal sea water* to permit a determination of the extent to which the effects produced are reversible. In the second place, a different method has been used for the quantitative measurement of the degree of retardation of the developmental processes. Instead of measuring the total number of cell divisions secured in a given time irrespective of whether they be the first, second, or third, the quantity here measured has been the time required for the first cleavage in each case. Furthermore, this has been done in such a way as to take into account

[1] *Amer. Journ. Physiol.*, LXXXII., 241.

not merely the mean time for all of the eggs but, approximately at least, the time for each individual egg. It is believed that data of this sort, while more troublesome to secure, are theoretically more significant and have a wider range of usefulness than those obtained by the other method.

Method.

Sea water, saturated with CO_2 from a Kipp generator, was used directly or was diluted as desired with oxygen-saturated sea water, with nitrogen-saturated sea water, or with ordinary sea water by siphoning together appropriate amounts of these solutions. It was then immediately siphoned into 75 cc. glass-stoppered bottles containing a few drops of a concentrated suspension of the newly fertilized eggs of *Arbacia punctulata*. As soon as the bottles were completely filled, they were tightly stoppered, shaken briefly, and placed in a bath of running sea water, which, with a few exceptions, varied in temperature not more than 0.5° C. for the duration of each experiment. For all the experiments during one season the range of temperature was 18.4° C. to 22.4° C., with a mean value of 20.3° C. In order to prevent a change in the solubility of the dissolved gases, it was deemed important always to have the CO_2-containing solutions at the temperature of the water-bath. Care was also taken to begin all the exposures of any one series as nearly simultaneously as possible, since a number of experiments not reported here seemed to indicate that sensitiveness to CO_2 may vary prior to the appearance of the first cleavage. In many of the experiments the bottles were inverted at five minute intervals to keep the contents well mixed, but other experiments in which this precaution was less rigorously observed gave essentially the same results.

At the time of setting up an experiment, samples of the solutions used were taken for estimations of the pH and dissolved oxygen. The former were immediately determined with the use of phenol red, brom thymol blue, brom cresol purple, and methyl red as indicators; the samples for the latter were kept at a constant temperature until Winkler determinations could be made. The results of the Winkler determinations are expressed as cc. of

oxygen per liter. Otherwise, the oxygen content is stated, as is the carbon dioxide content, in terms of percentage saturation. Assuming the applicability of Henry's law to gases in solution, it may be said that when 20 cc. of oxygen-saturated sea water is added to 80 cc. of CO_2-saturated sea water, the resulting solution contains CO_2 at 80 per cent. of saturation value and oxygen at 20 per cent. of saturation value. Since in adding the saturated solutions to the eggs they must experience a small interchange of gases with the air, a solution which was initially free of oxygen is referred to as having a trace of oxygen, while a CO_2-saturated solution is represented as having a CO_2 content of "100—" per cent. Wherever the tension of CO_2 is expressed in mm. Hg the value given is merely an approximate one, calculated for purposes of comparison with the work of other investigators on the assumption that the tension of carbon dioxide in a saturated solution is 760 mm., minus the vapor pressure of water at the temperature in question.

As already mentioned, the reversibility of the effects of CO_2 was determined by returning the eggs to sea water for development after the desired periods of exposure. Following a rinsing in sea water, the eggs were placed in small Pyrex beakers containing sea water to a depth of about 1.5 cm. Samples of the eggs were removed from the beakers at various times and were preserved for subsequent observation by the addition of a weak solution of formalin in sea water. The fixed eggs were placed in a large hanging drop where, if free from debris, they tended to settle in rows, which simplified the task of determining the percentages of eggs which had undergone the first cleavage. These values, found from time to time after a given exposure, give, when plotted against the minutes after fertilization, a curve which will be referred to as the cleavage curve for that particular exposure. The characteristic S-shape of this curve is related to the variability of the eggs themselves in the manner discussed by Loeb and Northrop (1917) and by Brooks (1918). The time required for cleavage in 50 per cent. of the eggs has in these experiments been used as the most convenient criterion of the cleavage rate, but other percentages could equally well be compared; the time in question can,

of course, be readily found by interpolation from the cleavage curve.

Results.

Before discussing the typical effects of carbon dioxide, it is necessary to rule out the possibility that oxygen lack might be a contributing factor in the results produced, since the method used in saturating the sea water with carbon dioxide causes at the same time a removal of oxygen. Although a complete lack of oxygen has been shown to stop the cleavage process in sea urchin eggs (E. B. Harvey, 1927), the present work indicates that even with an extensive reduction in oxygen tension cleavage is able to continue—and at a rate but little slower than normal. The results of the thirty minute exposures to low tensions of this gas, representing but 14 per cent. to 18 per cent. of those available for the controls, are given in Table I. and show that the cleavage time under these conditions was delayed but a few minutes.

Table I.

The Effect upon Cleavage of Thirty Minute Exposures to Low Oxygen Tensions.

Solution.	cc. of Oxygen per Liter.	Minutes Required for 50 per cent. Cleavage.
Sea water	5.6	60.5
Nitrogen-saturated sea water	0.76	68.5
Sea water	5.48	62.5
Nitrogen-saturated sea water	0.97	64.2

A direct comparison of the effects of low oxygen tension and of high carbon dioxide tension has been made in another experiment, in which each of four portions of egg suspension was exposed for thirty minutes to one of the following solutions:

	pH.	cc. of Oxygen per Liter.
1. Sea water	—	5.15
2. Sea water + oxygen + CO_2 (60% saturated)	5.3	5.56
3. Sea water + nitrogen + CO_2 (60% saturated)	5.3	1.26
4. Sea water, saturated with nitrogen	—	0.55

It will be evident that solutions 3 and 4 were low in oxygen as compared with solutions 1 and 2, while solutions 2 and 3 were high

in CO_2 as compared with solutions 1 and 4. The first cleavage appeared as shown in Fig. 1, where it will be seen that the shortest cleavage time occurred with the sea water control, represented by Curve 1. A lowering of the oxygen tension to slightly over one tenth of the normal value retarded the cleavage time but 4 minutes

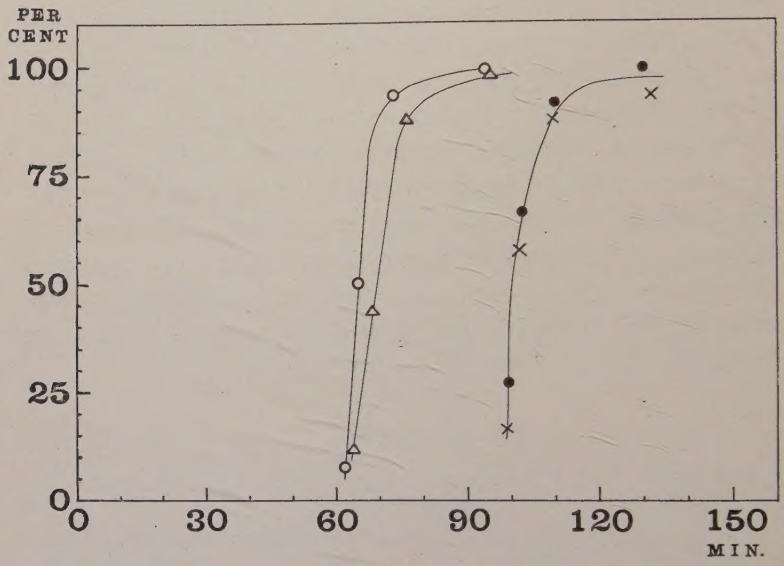

FIG. 1. The relative effectiveness of low oxygen tensions and high CO_2 tensions in delaying the first cleavage of *Arbacia* eggs. Exposures were of thirty minutes' duration. Temperature, 19.8°–20.2°. Abscissa = time, in minutes, after insemination. Ordinate = percentage of eggs showing the first cleavage.

	Symbol.	cc. Oxygen. per Liter.	CO_2.
Curve 1	○ Circles	5.15	
Curve 2	× Crosses	5.56	60%
Curce 3	● Dots	1.26	60%
Curve 4	△ Triangles	0.55	

(Curve 4). On the other hand, an increase of the CO_2 content to give pH of 5.3 was sufficiently great to retard the cleavage time 36 minutes, irrespective of the oxygen present, since a single curve serves to represent cleavage in the two solutions which, although showing more than a 4:1 difference in oxygen content, had the same carbon dioxide content. Since from such experiments as

the foregoing the cleavage process is seen to be but little affected by oxygen deficiency over a wide range, the more striking effects observed with CO_2, about to be described in more detail, may justly be attributed to an action of carbon dioxide in which incidental oxygen lack plays little or no part.

Such being the case, the solutions used in the subsequent experiments were simply mixed in a large graduated cylinder and immediately added to the eggs. Siphons were not used nor were Winkler determinations made. Speed in setting up the experiment was important, and in cases where a considerable number of bottles were to be filled, this method had the advantage of expediency. Where the sea water used was saturated with CO_2 (i.e. "100—" per cent. CO_2, with a trace of oxygen) it seems probable that sufficient oxygen must have entered the solution from the air to prevent any retardation of cleavage from oxygen lack, since a 4:1 mixture of CO_2-saturated and oxygen-saturated sea water (*i.e.* 80 per cent. CO_2 and 20 per cent. oxygen) gave practically the same result. In fact, the results following "100—" per cent. CO_2 were very similar to those of various tensions down to as low as about 30 or 40 per cent. CO_2. Inasmuch as most of the work here reported is not concerned with more than a semi-quantitative estimate of the gases, it is believed that the method is sufficiently accurate.

In striking contrast to the extensive diminution in available oxygen which the eggs seem to tolerate is the effect upon cleavage of even very small amounts of carbon dioxide. The repression of cleavage which occurs when, in the laboratory, the eggs are subjected to overcrowding is a familiar example of the effects which may be produced simply by the CO_2 which arises from the metabolism of the cells themselves. Experimentally, sea water containing as little CO_2 as 10 per cent. of saturation value was found, after an exposure of twenty minutes to delay cleavage twelve and one half minutes, while exposures of equal length to 30 per cent. and 40 per cent. CO_2 were found to delay cleavage a longer time—about twenty-three minutes. Apparently this latter value of 40 per cent. saturation or 300 mm. Hg represents practically a complete suppression of cleavage since it is approximately the same value as is obtained with higher tensions.

Table II.

Percentage of Eggs Cleaved during Exposure to CO_2.

Experiment.	No. 130.	No. 131.	No. 129.	No. 120.
Temperature	22.3°	21.7°–22.1°	21.3°–22.4°	21.2°–21.6°
Per cent. CO_2	15%	20%	30%	80%
pH	*6.25*	*6.2*	*5.95*	*5.2*
Exposures (in minutes):				
80′	10.9%	0%		
90′	27.7%			
100′		1%	0%	
120′			0%	
140′	56.8%			
160′			0%	
200′			6.8%	
300′			43.8%	
640′				0%

It is evident from Table II. that with less than 30 per cent. saturation the repression of cleavage is not complete, but only partial. At a value of 30 per cent., repression is almost complete, since only 7 per cent. and 44 per cent. of the eggs were able to cleave in three and one third and five hours respectively. It will be shown later in the paper that the effects of 30 per cent. and 40 per cent. CO_2 are so similar to those produced by greater amounts, that it is reasonable to suppose that at a saturation of 40 per cent. and over, representing a tension of 300 mm. Hg or more, the suppression of cleavage is practically complete. Therefore not only can very small amounts of CO_2 in the surrounding medium cause an appreciable delay in cleavage, but also its maximum action in suppressing cleavage is approached at values far below saturation.

Notwithstanding the prompt and extensive check upon cleavage which carbon dioxide produces, the reversibility of its effects upon returning the eggs to sea water serves to indicate that the action has been of a narcotic rather than of a purely toxic nature. After exposures of twenty minutes to sea water practically saturated with CO_2 there are usually no abnormalities in cleavage, and larvæ develop which show little or no difference from the normal controls. Following longer exposures, abnormalities appear, although they are relatively few in number with exposures of less than an hour. In spite of the presence of many abnormally

cleaved eggs, a few ciliated larvæ have been found to develop after an exposure of two and one half hours, and the first cleavage has made its appearance in 95 per cent. to 100 per cent. of the eggs subjected to very nearly 100 per cent. CO_2 for this length of time, though relatively few normal larvæ were obtainable after such long exposures. In one experiment—that illustrated in Table III.—exposure of the eggs to 80 per cent. CO_2 for over ten hours still permitted reversibility of the cleavage process to the extent that, six hours after the eggs had been returned to sea water, 88 per cent. were found to have divided, although development was very abnormal and went no farther than the first few cleavage stages. Apparently the process of nuclear division is an extremely powerful one and, as has been observed by others, can persist even when the cell itself is unable to divide.

Fig. 2 is a typical illustration of the series of cleavage curves which are obtained with various lengths of exposure to a relatively high tension of carbon dioxide. It will be observed that the first cleavage ultimately occurs in practically all of the eggs, even after prolonged exposures. The relation of the exposure time to the total retardation is a matter of some importance in indicating the nature of the observed effects. If the narcosis produced by carbon dioxide were complete and were followed by instantaneous recovery the resulting retardation of cleavage, as compared with normal controls, should be exactly equal to the time of exposure. Incomplete narcosis, on the other hand, would tend to shorten, and a more gradual recovery, to lengthen, the period of retardation. Theoretically, a combination of these two effects might conceivably, under a given set of conditions, result in a net retardation exactly equal to the period of the exposure, but it is believed that such a balancing of effects could not account for the results about to be described. Since with all tensions above approximately 30 per cent. saturation the relation of time of exposure to total retardation is essentially the same, it is difficult to imagine that the CO_2 tension should, above this point, either be without effect on a measurable rate of development or that a decrease in this rate should always be followed by a correspondingly increased rate of recovery. The reasonable interpretation of the

facts is that development is practically suppressed by the higher tensions of CO_2. The correctness of this view is also indicated by the fact that cleavage was not obtained during prolonged exposures to such solutions.

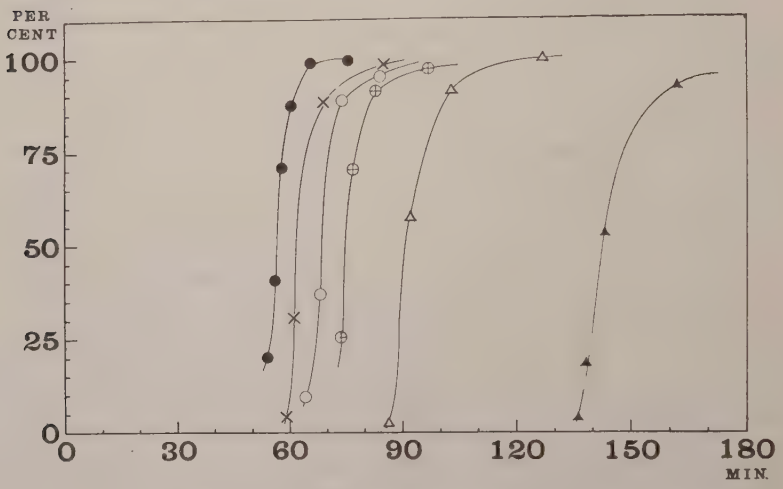

FIG. 2. The times required for the appearance of the first cleavage of *Arbacia* eggs following various lengths of exposure to sea water 80 per cent. saturated with CO_2. Temperature, 21.2°–21.6°. Abscissa = time, in minutes, after insemination. Ordinate = percentage of eggs showing the first cleavage.

Symbol.	Length of Exposure.
● Dots	0 minutes
× Crosses	5 minutes
○ Circles	10 minutes
⊕ Crosses in circles	20 minutes
△ Hollow Triangles	35 (?) minutes
▲ Solid Triangles	80 minutes

For each of the six curves the final value, omitted from lack of space, was 100 per cent., except in the case of the twenty minutes exposure, where it was 98 per cent. There have also been omitted the cleavage curves following exposures of 160, 320, and 640 minutes, which ultimately showed cleavage in 97 per cent., 99 per cent., and 88 per cent. of the eggs respectively. Table III. gives the delay in 50 per cent. cleavage corresponding to each cleavage curve of the complete series.

The relation between time of exposure and retardation of development is shown for a typical experiment in Table III., the values for 50 per cent. cleavage having been taken from the curves in Fig. 2.

Table III.

The Delay in 50 per cent. Cleavage Caused by Various Exposures to 80 per cent. CO_2.

(Experiment illustrated in Fig. 2.)

Exposure Time (in Minutes).	Delay in 50 per cent. Cleavage (in Minutes).
0	—
5′	5′
10′	12′
20′	18′
35′ (?)	34′
80′	86′
160′	186′
320′	370′
640′	750′

It will be observed that with the shorter exposures the recovery of the cleavage process may occur with great rapidity, the delay being but little more than the time corresponding to the period of exposure. Consequently, it has very often been found possible, in performing experiments, to predict the beginning of cleavage with a fair degree of accuracy simply by adding the exposure time to that required for the beginning of cleavage in the normal. As exposures become longer, the greater discrepancy between the exposure time and the delay of cleavage is wholly in one direction —that of prolonging the cleavage time. A further analysis of the data shows even in this respect a simple relation which may best be brought out by plotting the time required for 50 per cent. cleavage against the time of exposure, as has been done in Fig. 3. It will be noticed that straight lines may be drawn through the points representing any given experiment. Those indicated in the figure have been calculated by the method of least squares, with the result that the slopes of all the lines are approximately equal. All may be represented fairly accurately by the equation

$$y = a + 1.18x,$$

where a = the cleavage time of the eggs in the absence of CO_2; x = the time of exposure, and y = the cleavage time of the exposed eggs. Further data are given in Table IV.

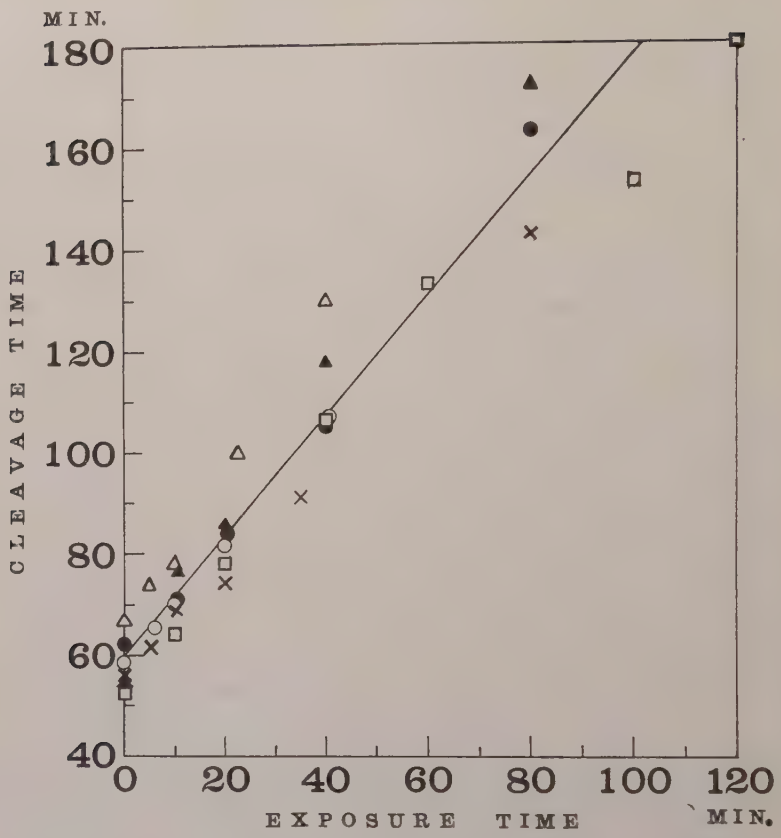

FIG. 3. The relation between the time required for 50 per cent. cleavage of *Arbacia* eggs and the duration of exposure to CO_2 at values from 30 per cent. saturation to practically complete saturation. Abscissa = minutes of exposure to CO_2. Ordinate = minutes required for cleavage in 50 per cent. of the eggs. Details are given in Table IV.

Symbol.	Experiment.
○ Circles	No. 119
× Crosses	No. 120
△ Hollow Triangles	No. 121
▲ Solid Triangles	No. 122
● Dots	No. 128
□ Squares	No. 129

(From lack of space, several values for exposures of over 120 minutes have not been included. These values were, however, used in calculating the equations the average of which—$y = 60.2 + 1.18x$—gives the straight line shown in this figure.) The slope of the curve would be unity, if recovery of the eggs were instantaneous upon their removal from CO_2.

Table IV.

Equations for the Retardation of 50 per cent. Cleavage in *Arbacia* Eggs in Sea Water 30 per cent. to "100 —" per cent. Saturated with CO_2.

Experiment.	CO_2 Content.	Oxygen Content.	Temperature.	Intercept.	Slope.
No. 119.....	"100 —"%	Trace	20.6°–21.2°	58.3	1.192
No. 120.....	80%	20%	21.2°–21.6°	52.6	1.182
No. 121.....	"100 —"%	Trace	20.4°–20.7°	71.	1.191
No. 122.....	"100 —"%	Trace	20.7°–20.8°	65.3	1.194
No. 128.....	40%	20%	20.8°–21.3°	59.35	1.23
No. 129.....	30%	20%	21.3°–22.4°	54.66	1.07
Mean Values.				60.2	1.18

(A preliminary experiment which showed a slope of 1.7 has been omitted from the average since this value differs widely from those subsequently obtained.)

The numerical value—1.18—of the slope of the lines in Fig. 3 shows that the retardation in cleavage produced by a given exposure is nearly, though not exactly, equal to the time of exposure. A value of unity would indicate exact equality. The fact that the slopes of the various lines are nearly the same is an indication of the general similarity, already mentioned, of the CO_2 effects at all tensions above 30 per cent. of saturation. The different values of the intercepts are without significance in this connection, since they represent merely the normal time of cleavage in those eggs which were not exposed to carbon dioxide.

Summary.

1. The first cleavage of the fertilized eggs of *Arbacia* is entirely suppressed, or practically so, in the presence of amounts of carbon dioxide greater than those corresponding to a 40 per cent. saturation of sea water or a tension of approximately 300 mm. Hg. In the presence of smaller amounts of carbon dioxide cleavage is possible, but is greatly delayed.

2. Since a very considerable oxygen deficiency causes only a slight delay in the cleavage process, the factor of oxygen lack is probably a negligible one in the results here described.

3. The effects of a complete suppression of the cleavage process

in sea water practically saturated with carbon dioxide are readily reversible up to exposures of twenty minutes. Beyond that point abnormalities may appear, though after exposures of two and one half hours 95 to 100 per cent. of the eggs ultimately divide.

4. The after effects of exposures of moderate length to carbon dioxide are comparatively slight, the delay in the first cleavage being only a little greater than the actual time of exposure. Mathematically, the relation

$$y = a + 1.18x$$

(where a = normal cleavage time; x = time of exposure; and y = cleavage time of the exposed eggs) has been found to describe fairly accurately the results obtained at 21.4° (± 1.°) with sea water from 30 per cent. saturation to almost complete saturation.

I am glad to have this opportunity to express my gratitude to Dr. M. H. Jacobs for his suggestion of this problem and for his continued interest in the progress of the work.

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CARBON DIOXIDE AS A NARCOTIC AGENT

II. The Effect of Carbon Dioxide upon the Contraction of Striated Muscle of the Frog

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The extensive and reversible inhibition of activity which carbon dioxide specifically produces in cells such as those of the developing echinoderm egg (Smith and Clowes, 1924; Haywood, 1927) suggests an inquiry as to its effectiveness upon striated muscle, which normally functions in the presence of more or less acid. The previous work in this field by Bottazzi (1916 a, b,) and others has been concerned with relatively large muscles, and under conditions where nervous elements might be present, and the resulting picture is therefore somewhat confused. The present investigation has as its object the further study of the action of carbon dioxide on striated muscle with the use of material believed to be somewhat more favorable than that previously employed. In particular, the attempt has been made to work with portions of muscular tissue so small and so thin as to favor the most rapid and complete exchange of gases possible with the surrounding solution and to use a method of recording contractions more delicate than that of the ordinary smoked drum. After trials of various methods it has been found possible to employ pieces of muscle about 6 mm. by 3 or 4 mm. in length and width respectively and 0.15 to 0.20 mm. in thickness and to measure contractions of 0.005 mm. The results here reported have been obtained under these conditions with the further precaution, usually neglected in the past, of the elimination of possible nervous factors by curarization.

Method. Electrodes, somewhat resembling those devised by Pratt (1917a) in their general arrangement, were constructed from two glass tubes, concentrically placed, and filled with a salt agar jelly into which dipped copper wires (fig. 1). The tubes were sealed into a hole in a flat block of paraffin (the electrode chamber) in such a way that their free ends partially formed the floor of a circular depression on the upper surface of the electrode chamber, which could be placed upon the stage of a

[1] Holder of the 1886 Fellowship of Mount Holyoke College (1926 1927) and of the Pepper Fellowship of the University of Pennsylvania (1926–1927).

microscope. The wires from the electrodes formed part of a secondary circuit, while the primary circuit consisted of a storage battery, a mercury key, and an induction coil arranged for repeated stimulation of a strength sufficient to evoke maximal contraction. In the later experiments, the mercury key was closed automatically by the pressure of a strip of spring steel upon which impinged an electrically driven cam. This method gave stimulations ranging in duration from 0.30 to 0.45 second and consisting of about sixteen to twenty-four individual stimuli, although during the course of any one experiment this entire range was probably never encountered. Stimuli of this short duration were ordinarily given at one minute intervals, a sufficiently generous recovery period to permit, in most cases, contractions of uniform amplitude.

Both the gastrocnemius and sartorius muscles of the frog were considered too large and thick to be useful for the problem at hand. However, the cutaneus pectoris muscle (Gaupp, 1896) of small specimens of *Rana pipiens* and of young *Rana catesbiana* was found to be admirably suited to the purposes of the work since the muscle is sufficiently small and thin to permit a rapid rate of diffusion, its fibers are parallel, and its attachments provide a convenient and firm point of support. After the muscle had been dissected out, as much of its surrounding membrane was removed as was easily possible, and for greater convenience the most lateral portion (usually about one-third or one-quarter) of the muscle was usually cut away. By means of fine needles the muscle could be held firmly in place in the electrode chamber containing Ringer's solution and its almost isometric contractions studied. Contraction was measured according to the method of Pratt (1917b) by observing with an ocular micrometer the excursions of a charcoal particle adherent to the muscle; in order to secure the maximum amplitude of movement, the particle was usually placed near one end of the muscle or upon the skin to which the muscle was attached. The depth of solution above the muscle was controlled by means of the focus of the microscope.

The Ringer's solution employed for most of the experiments was of the usual formula (Bayliss, 1924) except that the NaH_2PO_4 and glucose were omitted. The pH was lowered to 7.1 or 7.2 by adding a small amount of expired air. For larger amounts of CO_2, gas was added from a Kipp generator. The pH values of the solutions used were determined colorimetrically with brom cresol green, brom cresol purple, brom thymol blue, and phenol red, and are given without correction for the salt error.

The procedure of an experiment consisted first, of obtaining a series of contractions in ordinary Ringer's solution as a control, following which Ringer's solution containing CO_2 was pipetted on the muscle. Consistent results were obtained by frequently renewing the CO_2-containing solution in large excess between contractions. After the desired length of exposure

to CO_2, during which time the usual rhythm of stimulation was given, recovery in ordinary Ringer's solution was similarly studied. Since different muscles could not well be compared, a series of experiments was made on each muscle, using the same marker throughout. A graphic representation of contraction has been made by erecting upon an arbitrary base-line a series of ordinates which show the relative amplitudes of a series of individual contractions, the distance between successive ordinates corresponding to the time interval between successive stimulations.

RESULTS. The general depressant effect of CO_2 upon cell activity manifests itself, in the case of skeletal muscle, by a reduction in the height of contraction which is both immediate and pronounced. The regularity in the amplitude of contraction which is present when the muscle is stimulated in ordinary Ringer's solution at once disappears when Ringer's solution saturated with CO_2 (pH 5.2) is substituted. Even after one minute of exposure a significant reduction in contraction can ordinarily be observed

TABLE 1

The effect of Ringer's solution, saturated with CO_2, in reducing contraction.

	NUMBER OF PREPARATIONS REPRESENTED	CONTRACTIONS AS PER CENT OF NORMAL CONTRACTION	
		After 1 minute of exposure	After 5 minutes of exposure
Range of values	27	95.5–42.2	57.7–3.8
Mean values	27	79.5	25.6
Modes	27	81.8	26.3

(Note: One preparation which showed only 10.5 per cent of contraction after one minute has not been included here.)

and by the end of five minutes the effect is very pronounced (fig. 3: *A*, *B*, and *E*). The extent of the diminution in contraction is given in table 1, which averages the mean values observed for each of twenty-seven different muscle preparations after one minute and five minutes of exposure. The average effect of CO_2-saturated Ringer's solution is thus to reduce contraction, even after only one minute, to about 80 per cent of its original value, and after five minutes, to about 25 per cent of the normal.

With longer exposures contraction continues to diminish, although at a decreasing rate. It is very nearly abolished by the end of ten or fifteen minutes, although a longer time is usually required before the response to stimulation entirely ceases. In a typical experiment it was found that the loss of contraction was almost complete by the end of ten minutes, but another ten minutes elapsed before stimulation absolutely failed to evoke a response. Even then, complete inhibition was not well maintained, and the very slight contractions which were later evoked from time to time seem

to indicate that the effect was not a progressive, highly toxic one, but rather a narcosis which was scarcely deeper than sufficed to stop contraction.

Although the tardiness with which this final reduction appears can very possibly be explained, in part, by small losses of CO_2, due to the method used, yet inspection of the contour of the record indicates it to be an exponential relation of the "die-away" type. This is shown by the fact that an approximately straight line is obtained by plotting the logarithms

Fig. 1. Diagram of vertical section of electrodes and electrode chamber. × 0.45. D = depression in electrode chamber. X = inner electrode. Y = outer electrode. Both X and Y are filled with salt agar jelly. R = rubber stopper holding X in place. B = stopper plugging X. WX and WY = wires emerging from X and Y respectively. S = microscope stage.

Fig. 2. The reduction of contraction of frog skeletal muscle at the end of five minutes' exposure to Ringer's solution containing varying amounts of carbon dioxide. Abscissa = CO_2 expressed as per cent of saturation value. Ordinate = Amplitude of contraction expressed as per cent of normal amplitude.

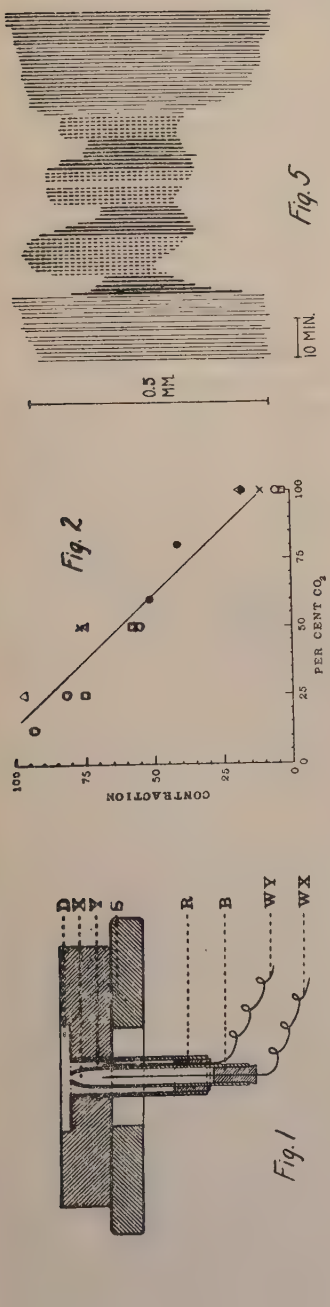
PERCENTAGE SATURATION	APPROXIMATE pH
100	5.2
80	5.3
60	5.4–5.5
50	5.6
25	6.1–6.2
$12\frac{1}{2}$	6.6

Temperatures: ● (Dots), 22° – 23.4°. × (crosses), 23.7° – 24°. ○ (circles), 24.2° – 24.8°. △ (triangles), 24.7° – 25.2°. □ (squares), 20.8° – 21°.

Fig. 3. The relative effectiveness of carbon dioxide, oxygen deficiency, and lowered pH in diminishing the amplitude of contraction of frog skeletal muscle. Light lines = contractions in ordinary Ringer's solution. Heavy lines = contractions in: A, B, E, CO_2-saturated Ringer's solution; C, nitrogen-saturated Ringer's solution; D, Ringer's solution acidified with HCl, giving a pH of 4.4. Temperature, 23°–24.1°.

Fig. 4. The relative effectiveness of CO_2-saturated solutions of varying $NaHCO_3$ content in diminishing the contractions of frog skeletal muscle. Changes in initial tension as well as in amplitude of contraction are shown. Light lines = contractions in ordinary Ringer's solution, M/420 with respect to $NaHCO_3$. Heavy lines = contractions in CO_2-saturated Ringer's solution, M/420 with respect to $NaHCO_3$ (pH 5.2–5.3). Dotted lines = contractions in CO_2-saturated solutions high in $NaHCO_3$. X = M/40 with respect to $NaHCO_3$ (pH 6.2). Y = M/15 with respect to $NaHCO_3$ (pH 6.6). Temperature, 20.2°–22.3°.

Fig. 5. The relative effectiveness of CO_2-saturated solutions of varying $NaHCO_3$ content in diminishing the contractions of frog skeletal muscle. Changes in initial tension as well as in amplitude of contraction are shown. Light lines = contractions in ordinary Ringer's solution. Heavy lines = contractions in CO_2-saturated Ringer's solution, M/420 with respect to $NaHCO_3$ (pH 5.2). Dotted lines = contractions in a CO_2-saturated solution, M/15 with respect to $NaHCO_3$ (pH 6.6). Temperature, 20.2°–22.2°.


D
X
Y
S
R
B
WY
WX
Fig. 1
CONTRACTION
100
75
50
25
0
0
25
50
75
100
PER CENT CO_2
Fig. 2
0.5 MM.
10 MIN.
Fig. 5
1 MM.
10 MIN.
A
B
C
0.5mm.
5min.
REST
D
E
Fig. 3
Fig. 4

of the height of contraction against the time. The slope of this line is such as to indicate that during the first six or eight minutes any given contraction is, on the average, approximately 0.8 as great as the one preceding it by one minute.

The action of concentrations of carbon dioxide below saturation value is less striking, but significant, and is usually manifested promptly. In figure 2 are charted five experiments which consist of two or more exposures, of five minutes each, to CO_2 of different concentrations, each exposure being followed by recovery in ordinary Ringer's solution. In two cases, Ringer's solution 25 per cent saturated with CO_2 (pH 6.1–6.2) was found to diminish contraction appreciably, and the intermediate values between this one and complete saturation indicate that the reduction of contraction is roughly proportional to the amount of CO_2 present.

As an indication of a narcotic rather than of a toxic action, the reversibility of the effects produced by carbon dioxide is of importance. The mere substitution of ordinary Ringer's solution for that containing CO_2 results invariably in some degree of recovery, notwithstanding the fact that no break has occurred in the rhythm of stimulation to allow a longer interval of rest. In general, the time required for recovery tends to vary inversely with the concentration of CO_2; for example, in one experiment recovery from practically 100 per cent saturated Ringer's solution required twenty-one minutes, while after an exposure of equal duration to 50 per cent CO_2 recovery was complete in thirteen minutes. Although the various muscle preparations have been found to show wide differences in their ease of recovery, yet after an exposure of five or ten minutes to a Ringer's solution saturated with CO_2 (pH 5.2) the effects of the gas can usually be completely reversed. The first minute after exposure is always found to give a conspicuous return toward the original amplitude of contraction. Complete recovery usually follows more gradually, but, once reached, is ordinarily well maintained. In general the same picture tends to hold true for somewhat longer exposures, such as those of twenty or thirty minutes. In such cases, however, the first minute following exposure gives a less conspicuous return of contraction, and subsequent recovery tends to be incomplete and less rapid. Recovery, even under these conditions, may be extensive; in one experiment an exposure of thirty minutes, which had practically abolished contraction for twenty-four minutes of that time, was followed by a return of contraction to 93 per cent of its original value; after the muscle was allowed to rest, without stimulation and in the absence of CO_2, for thirty-seven minutes, during which time recovery became practically complete, a further exposure of forty minutes was imposed and, in spite of the fact that stimulation evoked but a slight response during thirty-five minutes of this time, a recovery of 87 per cent was finally obtained. Even very long exposures to CO_2 permit some de-

gree of recovery. During one exposure of one and one-half hours there was absolutely no response to stimulation for the last thirty-eight minutes, and yet, after the substitution of ordinary Ringer's solution, a very slight contraction was evoked in two minutes, and by the end of two hours a 40 per cent recovery had been obtained.

In the experiments so far discussed, only the total contraction of the muscle has been measured. It is possible, however, by a slightly different method of graphic representation to take into account the initial tension of the muscle as well. In such cases the electrode chamber was held immovable during the course of an experiment, and both the excursion of the charcoal marker and its point of departure were determined with reference always to some one point on the micrometer scale; a corresponding plotting of the amplitude of contraction with reference to this same point thus serves to represent the changes in initial tension, as with the usual smoked drum record, by a changing base-line. When changes in the initial tension are thus taken into consideration, an increased contracture is seen to occur during exposure to CO_2 (fig. 4). This finding confirms Bottazzi (1916a) in showing that contracture is to some extent responsible for the resulting diminution in contraction. It will be observed that not only is contracture slow in disappearing, but it may even continue to increase for two or three minutes after the removal of the muscle from the CO_2-containing solution. The latter fact makes it unlikely that the diminished amplitude observed during exposure might be the result of an increasing initial tension, since after exposure the amplitude of contraction immediately returns toward its original value at the same time that the initial tension is still showing a slight increase,—an observation which may possibly be of interest in relation to Bottazzi's view that tonic contraction is independent of clonic contraction.

A question of much importance is the manner in which carbon dioxide produces its narcotic effects. Lack of oxygen in CO_2-saturated solutions can readily be ruled out under the conditions prevailing in these experiments, since solutions saturated with nitrogen and administered in an exactly similar manner as were the CO_2-saturated solutions have very little effect (fig. 3*C*). This result was of course to be anticipated from the observation of Fletcher (1902) that muscle may continue to contract for a long time in the absence of oxygen. A second possible factor is connected with the chemical nature of carbon dioxide. Unbuffered solutions containing considerable quantities of this substance have a more or less strongly acid reaction, and even when buffered to the point of actual alkalinity they may nevertheless produce an intracellular acidity (Jacobs, 1920a, b). One possibility, therefore, is that a change in pH either of the cells or of their surroundings is concerned. That the pH, as such, of the surroundings is of little importance is indicated by the fact that a decided

lowering of the pH of the solution by a "non-penetrating" acid such as HCl is almost without effect. It will be seen from figure 3*D* that the entire absence of inhibitory effects during a ten minute exposure to a CO_2-free solution of a pH of 4.4 offers a striking contrast to the marked decrease in contraction produced by an exposure, half as long, to a CO_2-saturated solution of pH 5.2. In no case could the negative results obtained from lowered pH and oxygen deficiency be due to a gradual decrease of sensitiveness of the muscle, since in this and other similar experiments CO_2 was shown to exert its characteristic action at both the beginning and the end of the experiment.

The effects of carbon dioxide on the internal pH of the cells is probably of much more importance than is the pH of their surroundings. The quantitative experiments of Smith (1925, 1926) indicate, in the case of "penetrating" acids, the importance of the concentration not only of the undissociated acid, but of its salt as well. To determine whether the presence of bicarbonate in a CO_2-bicarbonate mixture affects skeletal muscle similarly, experiments of the following type were devised. A solution was made up resembling the Ringer's solution ordinarily used except that for some of the NaCl there was substituted an equimolecular amount of $NaHCO_3$. After saturation with CO_2, the resulting solution, as compared to a CO_2-saturated Ringer's solution, was of the same CO_2 content and of the same osmotic pressure, but differed as to pH and as to bicarbonate and chloride content. The results of a typical experiment, illustrated in figure 4, show the reduction of contraction in five minutes to be, in this case, the following:

SOLUTION	pH UPON SATURATION WITH CO_2	CONTRACTIONS AS PER CENT OF NORMAL
Ringer's	5.2–5.3	44.0
Ringer's; M/40 with respect to $NaHCO_3$	6.2	64.0
Ringer's; M/15 with respect to $NaHCO_3$	6.6	72.0
Ringer's	5.2–5.3	31.0

More striking results of the same kind were obtained when the experiment was somewhat varied, as in figure 5, where the substitution of Ringer's solution high in bicarbonate, but saturated with CO_2, was found to allow some degree of recovery. It will be seen that, after this incomplete recovery obtained in the bicarbonate solutions, a second administration of Ringer's solution, saturated with CO_2, caused contraction again to be reduced. It thus seems evident that in the case of skeletal muscle as well as cardiac muscle (Smith, 1926) diminution of contraction is not determined solely by the concentration of the free acid. The modification of the characteristic effects of carbon dioxide by the addition of bicarbonate suggests the probability that the narcotic action of the latter substance is

related in some way to its ability to alter the internal reaction of the cell.

SUMMARY

1. An electrode chamber has been designed which permits the medium surrounding a very small muscle to be changed frequently without material alteration of contact with the electrodes.

2. In Ringer's solution saturated with CO_2 (pH 5.2) the contraction of the cutaneus pectoris muscle of the frog is on an average reduced in one minute to 80 per cent of the original value and in five minutes to approximately 25 per cent. In ten or fifteen minutes contraction may be very nearly abolished. The relation of the heights of contraction at successive intervals of one minute is roughly an exponential one.

3. Complete recovery occurs after exposures of ten minutes or less to CO_2-saturated Ringer's solution; following exposures of twenty to forty minutes recovery tends to be incomplete yet still extensive, and an appreciable recovery has been obtained after an exposure of one and a half hour.

4. With amounts of CO_2 corresponding to less than 100 per cent saturation the reduction in contraction is proportionally less.

5. The effects produced by CO_2 cannot be imitated by lack of oxygen nor by a lowering of the pH of the surrounding solution by a non-penetrating acid such as HCl.

6. The addition of bicarbonate in increasing amounts to a CO_2-saturated solution does not abolish the typical CO_2 effects, but diminishes them considerably in a manner similar to that reported by Smith (1925, 1926) for *Echinarachnius* eggs and for the turtle auricle. This effect of bicarbonates suggests that the narcotic action of CO_2 may be associated in some way with its ability to produce intracellular acidity.

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